Capillary Electrophoresis as a Tool for Evaluating Lactic Acid Production from Sorghum

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ABSTRACT

Sorghum is an underutilized resource for the production of bioindustrial chemicals like lactic acid. Capillary electrophoresis (CE) was tested to monitor the fermentation process, i.e., quantify the amount of lactic acid and by-products in the fermentation broth using phosphate buffer pH 6.25 containing the electroosmotic flow modifier cetyltrimethylammonium bromide (CTAB). High levels of calcium carbonate were required during fermentation to stabilize the pH and these caused considerably prolonged migration times in CE. A 1:10 dilution of the samples with water was the best way to reduce salt load and thus conductivity in the sample plug, and thus to eliminate the problem of prolonged migration times. Further improvements were achieved by rinsing the capillary with HCl and water after each run, rather than NaOH and water. HCl might more efficiently remove Ca^{2+} ions from the capillary surface. The fermentation broth studied was based on liquefied sorghum inoculated with *Rhizopus oryzae*. The main product was lactic acid (24.60 ± 0.56 g/L) and a significant by-product was fumaric acid (1.07 ± 0.04 g/L).

Interest in the use of renewable resources for the production of bioindustrial chemicals and fuels is increasing (Bastioli 2001). One industrial chemical that can be produced from renewable resources is lactic acid. Lactic acid is used in the food industry as a flavor compound, preservative, and to acidify products (Wee et al 2006; John et al 2007). Lactic acid can also be used to produce polylactic acid (PLA), lactate esters, propylene glycol, propanoic acid, acrylic acid, and other chemicals. PLA can be made into biodegradable plastic material. The world market for PLA is expected to increase rapidly in the future (Wee et al 2006).

Lactic acid can be produced either through chemical synthesis or through a fermentation process (Wee et al 2006; John et al 2007). A major potential source of such renewable resources to produce lactic acid through fermentation would be agricultural-based materials such as cereal grains. Through an enzymatic process, starch from cereal grains can be broken down to sugars that in turn can be used to produce lactic acid from a fermentation process.

One grain with high potential for the production of biobased products is sorghum. Sorghum is a drought-resistant, heat-tolerant cereal grown primarily in Asia, Africa, and the United States. Sorghum production ranks third among cereal crops in the United States and is currently used mainly as animal feed, although its use in fuel ethanol production is increasing. Recent research has demonstrated that lactic acid can be produced from sorghum (Zhan et al 2003).

The production of biochemicals from cereal grains such as sorghum faces technological and economic challenges. Many factors influence the production of biochemicals from cereal crops including physical and chemical properties of the grain and how the grain is processed (Corredor et al 2006; Wu et al 2007). Thus it is important to have the ability to monitor the production process and characterize the final compounds produced including by-products.

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Many analytical techniques have been used to monitor fermentation processes and characterize fermentation products. High-performance capillary electrophoresis (HPCE) was used successfully to separate organic acids from numerous samples with good resolution and rapid separation times (Klampfl et al 2000; Galli et al 2003; Wang et al 2003; Galli and Barbas 2004). HPCE utilizes inexpensive silica capillaries to perform separations. Due to the small size of the capillaries, less solvents or buffers are used in the process. Thus HPCE may provide an inexpensive and rapid technique for monitoring the fermentation of sorghum to lactic acid and for characterizing by-products. The goals of this project were to optimize a HPCE method specifically to evaluate the production of lactic acid from sorghum. This was an especially challenging sample matrix to work with due to the high levels of calcium carbonate which is added to stabilize the pH of the fermentation broth (Hang 1989; Zhan et al 2003).

MATERIALS AND METHODS

HPCE Separations

A Beckman PACE 2100 (Fullerton, CA) was used for all HPCE separations. Separations were conducted using a 0.5*M* phosphate buffer pH 6.25 containing 0.5 m*M* cetyltrimethylammonium bromide (CTAB) with direct UV detection at 200 nm as described by Galli and Barbas (2004). Briefly, an uncoated fused silica capillary 57 cm (L_d 50 cm) × 50 μm i.d. was used for all separations. Samples were injected by pressure (0.035 bar) for 15 sec and separated at –10 kV with a capillary temperature of 25°C. The capillary was rinsed with separation buffer for 5 min at 1.4 bar before each separation. Three different postseparation rinsing protocols were tested: 0.1*M* NaOH (3 min) followed by water (3 min) as used in the above method; a 0.1*M* HCl rinse (3 min) followed by deionized water (3 min); and a deionized water (3 min) only rinse.

The optimized method included a startup sequence at the beginning of each day (rinsing at 1.4 bar with 0.1*M* HCl, 3 min; water, 3 min; separation buffer, 15 min; followed by the application of 15 kV for 5 min). At the end of each day before shutdown, the capillary was rinsed with 0.1*M* HCl (10 min) followed by water (20 min). For long-term storage, it was subsequently blown dry with nitrogen.

Sample and Standard Preparation

Lactic acid was produced as described in Zhan et al (2003). Briefly, 15 g of sorghum meal (db)/100 mL of fermentation broth was used and various minerals and urea (nitrogen source) were added. The sorghum starch in this medium was liquefied with thermostable α-amylase, and then the media were sterilized and

inoculated with a preculture of *Rhizopus oryzae*. To maintain the pH, 6 g of calcium carbonate/100 mL was added in two portions. After the fermentation (72 hr at 33°C), the fermentation broth was brought to boil, cooled, and filtered through coarse filter paper (Fisherbrand P8, Fisher Scientific, Pittsburgh, PA). Subsequently, samples were diluted 1:10 with deionized water and centrifuged for 3 min at 16,000 × *g* before injection. Lactic acid and fumaric acid standards used to construct calibration curves were carefully prepared in such a way to mimic the fermentation process so that the sample matrix of the standards closely matched that of fermentation samples. For this purpose, dilution series of lactic acid and fumaric acid were prepared. From each dilution, an aliquot (10 mL) was taken and mixed with 0.6 g of calcium carbonate, heated to 100°C, filtered, diluted 1:10 with deionized water, and then centrifuged 3 min at 16,000 × *g*. For the determination of detection limits (concentrations resulting in peak heights corresponding to three times the average baseline noise), stock solutions of lactic, fumaric, acetic, and citric acid were first stepwise diluted 1:10 to determine the approximate limit. For fine-tuning, solutions of these acids around the estimated limit concentrations were prepared as described for the lactic and fumaric acid standards, taking into account the 1:10 dilution during sample preparation after boiling with calcium carbonate. The concentrations reported as detection limits are therefore the true detection limits of this method for real fermentation samples, for which a subsequent 1:10 dilution is required due to the high calcium concentrations.

RESULTS AND DISCUSSION

Galli and Barbas (2004) successfully separated short chain organic acids from coffee using a pH 6.25 phosphate buffer containing the electroosmotic flow (EOF) modifier CTAB. CTAB is a cationic surfactant that binds to the negatively charged capillary wall and modifies EOF. With this buffer system, these authors were able to resolve 17 organic acids. Thus, we selected this method to explore the use of HPCE for monitoring the fermentation of sorghum to lactic acid.

Initial separations of organic acid standards produced high-resolution separations; however, migration times were not consistent when a sample of fermentation broth was repeatedly injected (data not shown). While many factors can contribute to migration time repeatability in HPCE, two of the major areas are the ionic strength of the sample matrix and the postseparation condition of the capillaries (Bean and Lookhart 2001).

In HPCE, the sample plug should have a lower conductivity than the background buffer as high levels of salt in the sample matrix can lead to heating of the sample plug, poor sample stacking, and poor repeatability (Bean and Lookhart 2001). Because the fermentation process required considerable amounts of calcium carbonate for neutralization (Zhan et al 2003) high levels of salt were present in the sample matrix.

A series of experiments was conducted to test whether the negative effect of the calcium carbonate in the sample matrix could be overcome. First, a lactic acid solution was made up with no added salt and injected at two concentrations ("as is" and a 1:10 dilution with deionized water) (Fig. 1). No migration time shifts were noticed between the lactic acid peaks from these injections. Next, calcium carbonate was added to the lactic acid solutions at the same levels as was present in the fermentation broth and again injected at two concentrations ("as is" and a 1:10 dilution with deionized water) (Fig. 1). In this case, the sample with the higher salt concentration (i.e. the "as is" nondiluted sample that had higher salt levels loaded into the capillary from the sample plug) had migration times that were shifted considerably from the diluted sample. The migration times of the diluted sample matched those of the pure lactic acid samples (Fig. 1). Finally, a fermentation sample was injected at two concentrations, again "as is" and one that had been diluted with water 1:10 (Fig. 2). The results were similar to those for the calcium carbonate added

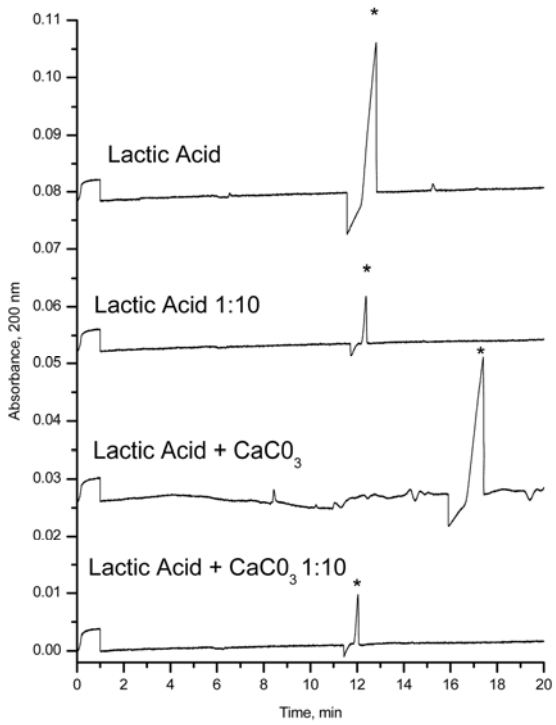

Fig. 1. Separation of lactic acid at two different concentrations with and without added $CaCO_3$. Run buffer was 0.5*M* phosphate buffer pH 6.25 containing 0.5 m*M* cetyltrimethylammonium bromide (CTAB). Lactic acid peaks marked with *.

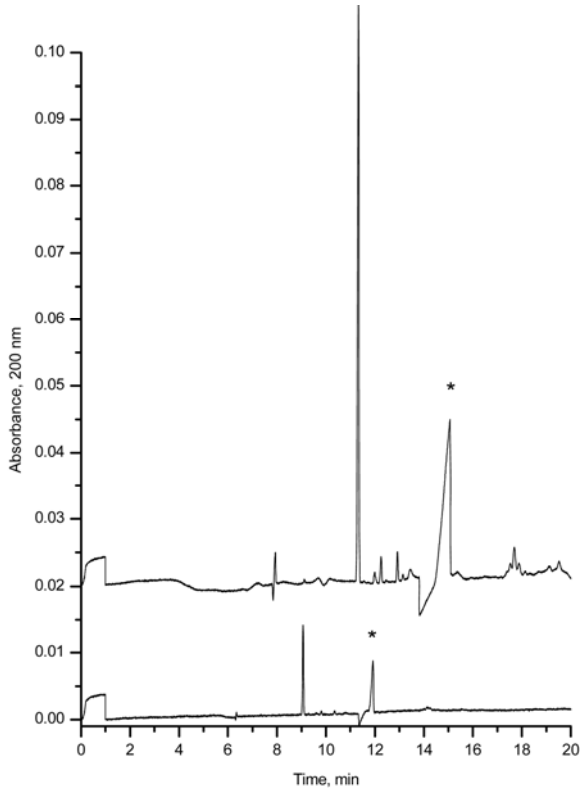

Fig. 2. Separation of fermentation broth undiluted and diluted 1:10 with deionized water. Run buffer as in Fig. 1. Lactic acid peaks marked with *.

samples, i.e., more salt in the sample plug resulted in a substantial migration shift from the other sample and the migration time of the lactic acid peak from the sample diluted to lower salt levels matched that of the pure lactic acid sample (compare Fig. 1 to Fig. 2). Thus it appeared that the calcium carbonate in the sample matrix and the amount of salt loaded into the capillary did influence the migration times of the analytes. To overcome this, samples were first diluted with deionized water (1:10) before analysis. This reduced the levels of salt being loaded into the capillary. Attempts to complex the calcium ions with EDTA or precipitate them from the samples before injection by adding acetonitrile or 1-propanol were not successful (data not shown). While it may be possible to use desalting columns to remove the calcium before injection, the dilution worked well and was inexpensive and simple.

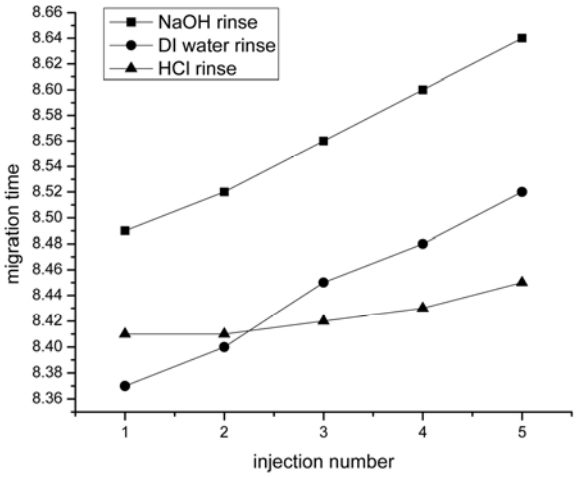

Fig. 3. Migration time stability of lactic acid under the different postseparation rinsing protocols.

To further optimize the repeatability of the method, three different postseparation rinses were evaluated using a 1:10 diluted fermentation sample. The first, as described in the original method, was a 3-min 0.1*M* NaOH rinse followed by a rinse of deionized water. Using this protocol, the migration times of the lactic acid peak varied by 0.7% (n = 5) but with a constant drift to longer and longer migration times (Fig. 3). Next, a rinse of only deionized water was tested and migration times of the lactic acid peak varied by 0.71% (n = 5) also with a tendency to drift to longer migration times. Finally, a rinsing procedure consisting of a 3-min rinse with 0.1*M* HCl followed by a 3-min rinse with deionized water, in which the lactic acid peak migration times varied by only 0.2% (n = 5), providing the lowest variability and the least tendency for migration times to drift. Thus, the HCl rinse was selected for routine use. While in the analysis of coffee, the use of a base rinse was necessary to remove sample components bound to the capillary wall (Jones and Jandik 1992), with our samples, the HCl performed better. This may have been due to the ability of HCl to leach minerals such as calcium from the capillary walls (Barrett et al 2001; Gómez and Sandoval 2008). With sample dilution and the HCl postseparation rinse, the method displayed excellent repeatability (Fig. 4).

To identify the organic acids present in the sorghum fermentation samples, standards of lactic acid, fumaric acid, acetic acid, citric acid, tartaric acid, and malic acid were analyzed and compared with a sample of fermentation broth (Fig. 5). In addition, a fermentation sample was spiked with each acid individually and analyzed (data not shown). These experiments revealed that the major components in the sorghum fermentation samples were lactic and fumaric acid. Citric acid was also present, but only in traces. The microorganism used in these experiments to produce lactic acid (*Rhizopus oryzae*) has been reported to also produce fumaric acid at varying levels (Oda et al 2002). Calibration curves for both lactic acid and fumaric acid were developed and showed

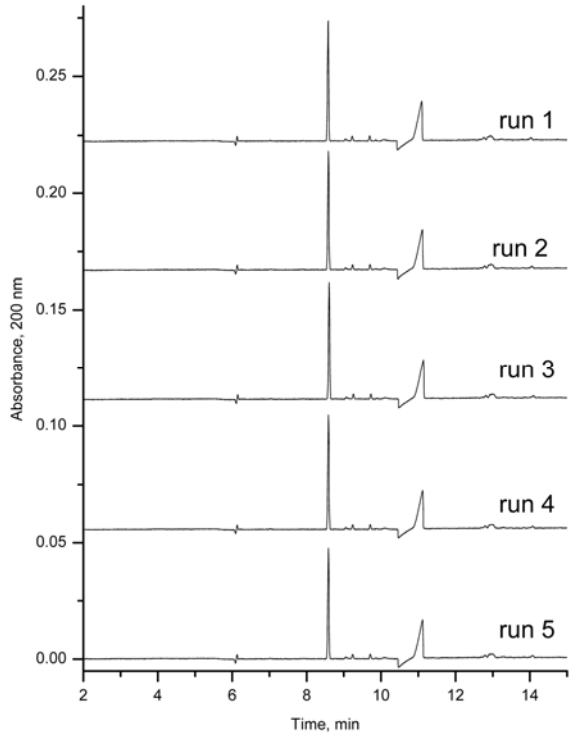

Fig. 4. Successive injections of fermentation broth with a post-separation rinse of 0.1*M* HCl and DI water showing the repeatability of the method. Sample was diluted 1:10 with DI water before injection and separated using 0.5*M* phosphate buffer pH 6.25 containing 0.5 m*M* cetyltrimethylammonium bromide (CTAB). Peaks at ≈8.5 min and ≈11 min correspond to fumaric and lactic acid, respectively.

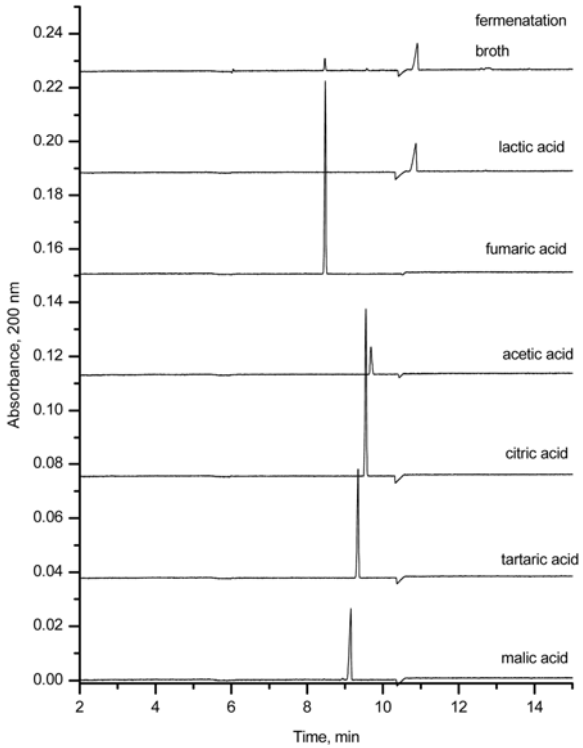

Fig. 5. Separation of fermentation broth and organic acid standards. Run buffer as in Fig. 1.

TABLE I
Amounts of Lactic Acid and Fumaric Acid in Five Successive Injections of Fermentation Broth (Fig. 4) Based on Calibration Curves (Fig. 6)

	Lactic Acid		Fumaric Acid	
Run	**Corrected Areas**	**g/L**	**Corrected Areas**	**g/L**
1	10,152	24.72	11,423	1.10
2	10,160	24.74	11,484	1.10
3	10,431	25.40	11,256	1.08
4	9,961	24.25	10,974	1.05
5	9,822	23.91	10,575	1.02
Average		24.60		1.07
Standard deviation		0.56		0.04
Coefficient of variation (%)		2.3		3.3

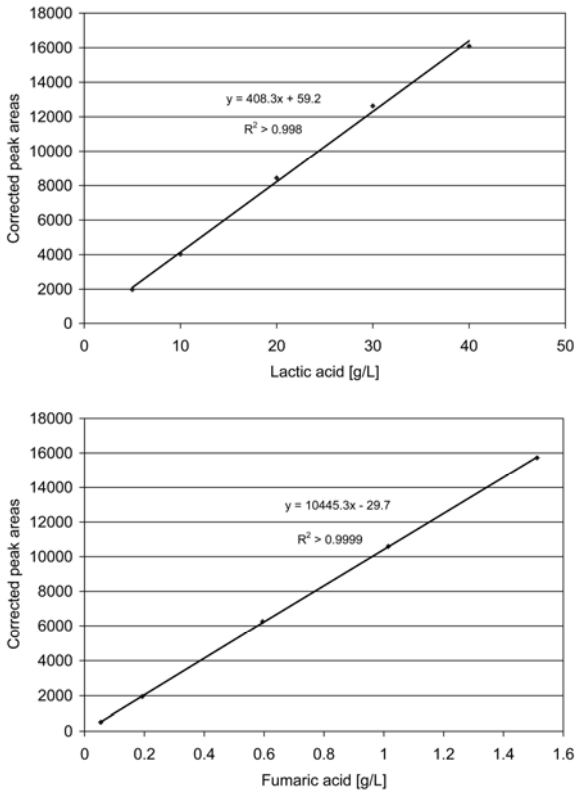

Fig. 6. Calibration curves for lactic acid and fumaric acid.

excellent linearity (Fig. 6). Thus, this HPCE method can be used to quantify the amount of these acids in sorghum fermentation samples. Using these calibration curves, lactic and fumaric acid in the fermentation broth (Fig. 4) were quantified. Based on these five successive separations, the quantitative repeatability of the method was verified (Table I). Coefficients of variation of ≈3% indicate good repeatability of the method on real world samples. When five separations were made within one week on different days each, the coefficients of variation were ≈6% for both lactic and fumaric acid (data not shown). This somewhat higher error was expected due to the longer time between measurements, and the need to run startup and shutdown cycles every day. We would recommend to run lactic and fumaric acid standards at least weekly to verify that no unacceptable changes occur over time.

Besides repeatability, the detection limits of the method are of interest. Four organic acids were selected for the determination of their respective detection limit: lactic, fumaric, acetic, and citric acid. While it may be important to detect the major and minor fermentation product (lactic and fumaric acid) early in the fermentation at low levels, citric acid was chosen because it was present in traces. Acetic acid could not be detected in the present fermentation broth. It is, however, a good indicator of a microbial contamination, as many types of microorganisms form it during

TABLE II
Detection Limits for Four Selected Organic Acids[a]

	g/L	10^{-3} mol/L
Lactic acid	0.62	6.9
Fumaric acid	0.010	0.086
Acetic acid	0.83	14
Citric acid	0.42	2.0

[a] Concentrations resulting in peak heights of 3× average baseline noise.

fermentation. Table II shows the detection limits for the four organic acids. The sensitivity of the method (based on molar concentrations) followed the order expected for UV detection. It was least sensitive to acetic acid (only a single carboxyl group present), followed by lactic acid (carboxyl and hydroxyl group), citric acid (three carboxyl, one hydroxyl group), and was most sensitive to fumaric acid (double bond and two carboxyl groups). Generally, the detection limits were slightly higher than those reported by Galli and Barbas (2004) for these acids, although in the same order of magnitude (increase < times 10). This was most likely caused by the required 1:10 dilution to reduce the salt load in the present study. Despite this finding it is noteworthy that all by-products (fumaric, acetic, and citric acid) can be detected at very low concentrations when compared with the concentration of lactic acid present in the ripe fermentation broth (Table I). It is also noteworthy that the prominent peak corresponding to fumaric acid is due to the high sensitivity of the method to this organic acid. In fact, it constitutes <5% of the amount (g/L) of lactic acid produced.

Zhan et al (2003) determined the amount of lactic acid by a simple titration. Although titration lacks specifity, under normal conditions, it provides sufficiently correct results because of the high excess of lactic acid. If, however, some contamination occurs, HPCE will quickly show the appearance of an unusual pattern of organic acids.

CONCLUSIONS

HPCE can be used as a routine method to evaluate the production of lactic acid and other organic acids in fermentation broths. It utilizes very small sample sizes, is rapid, specific, sufficiently repeatable, and capable of very high resolution, making it well suited as a tool for monitoring the fermentation of cereal grains. Such new techniques to monitor the fermentation process and characterize the fermentation products could enhance the production of biobased materials from fermentation and help to prevent losses due to contamination or other production mistakes.

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